## PCT

| (51) International Patent Classification 6 : C12N 15/35, A61K 48/00 | A1 | (11) International Publication Number: WO 97/40163 |
|---|---|---|
| | | (43) International Publication Date: 30 October 1997 (30.10.97) |

(21) International Application Number: PCT/EP97/01943

(22) International Filing Date: 18 April 1997 (18.04.97)

(30) Priority Data:
96106217.1      19 April 1996 (19.04.96)    EP
(34) Countries for which the regional or
     international application was filed:    DE et al.

(71)(72) Applicant and Inventor: COLPAN, Metin [TR/DE]; Uh-land Strasse 5, D-45219 Essen (DE).

(72) Inventors; and
(75) Inventors/Applicants (for US only): SCHORR, Joachim [DE/DE]; Rubensweg 15, D-40724 Hilden (DE). BAKER, Henry, J. [US/US]; 1804 Creekwood Trail. Auburn, AL 36830 (US). SMITH, Bruce, F. [US/US]; 642 Woody Drive, Auburn, AL 36832 (US).

(74) Agent: VOSSIUS & PARTNER; Siebertstrasse 4, D-81675 München (DE).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
     With international search report.
     Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NUCLEIC ACID VACCINATION FOR PARVOVIRAL INFECTIONS

(57) Abstract

     The general field of the invention is a method for nucleic acid vaccination of animals to protect them from parvoviral infections. This invention is more particulary related to the preparation and use of parvoviral DNA and its administration to dogs, cats and mink so as to induce an immune response that can protect these animals from disease caused by virulante parvovirus. Nucleic acid immunogens are designed to include the antigenic portions of the parvoviral genome which are incorporated into bacterial plasmids. These plasmids produce the desired parvoviral gene product when introduced into host cells by transfection. Host cells transfected with the parvoviral immunogen expressing plasmids produce a stream of antigenic proteins to which the host immune system will mount a protective immune response.

# NUCLEIC ACID VACCINATION FOR PARVOVIRAL INFECTIONS

The general field of the invention is a method for nucleic acid vaccination of animals to protect them from parvoviral infections. This invention is more particularly related to the preparation and use of parvoviral DNA and its administration to dogs, cats and mink so as to induce an immune response that can protect these animals from disease caused by virulent parvovirus. Nucleic acid immunogens are designed to include the antigenic portions of the parvoviral genome which are incorporated into bacterial plasmids. These plasmids produce the desired parvoviral gene product when introduced into host cells by transfection. Host cells transfected with the parvoviral immunogen expressing plasmids produce a stream of antigenic proteins to which the host immune system will mount a protective immune response.

Parvoviruses are a family of closely related small DNA viruses composed of a protein capsid containing single stranded DNA. Parvoviruses cause various diseases in a variety of mammalian species. Feline panleukopenia virus, mink enteritis virus and canine parvovirus are host range variants of the feline parvovirus subgroup and share more than 98% DNA homology (Martyn et al, J. Gen. Virol. 71 (1990) 2747-2753). Canine parvovirus is a relatively new pathogen of all canids, having been first recognized in the late 1970's as the causative agent of a world wide pandemic of highly fatal gastroenteritis. This virus is theorized to be a host range mutant of feline parvovirus. When nucleotide sequences of feline and canine parvoviruses were compared, 31 base changes were identified resulting in changes in just 9 amino acids (Martyn et. al., 1990). Six of these amino acid changes were in the major capsid genes VP1 and VP2. The canine specific antigenic epitope is determined by a single amino acid difference from feline panleukopenia virus. Further genetic mapping has recently confirmed the time of origin of canine parvovirus, reinforced the theory of its origin from feline parvovirus, and indicated a continued evolution of the virus in the field strains now being isolated (Truyen et. al., J. of Virology 69 (8) (1995), 4702-4710).

The two nucleocapsid proteins, VP1 and VP2 are expressed from the same RNA
with VP2 resulting from an in-frame ATG codon within the VP1 open reading frame.
VP2 is expressed at levels nearly 10 fold higher than VP1 indicating that the
internal start codon is more efficiently recognized as such by the translational
apparatus (Turiso et. al., J. of Gen. Virol. 72 (1991), 2445-2456). Epitope mapping
experiments have demonstrated that all of the antigenic epitopes generating
neutralizing antibody lie within VP2 (Turiso et. al., 1991, loc. cit.). These include the
first 16 amino acids of VP2 (Langeveld et., J. of Virology 68(7) (1994), 4506-4513;
Casal et. al., J. of Virology 69 (11) (1995), 7274-7277).

Immunization remains the primary mechanism by which humans and animal
species are protected against the scourge of infectious disease. The recent trend
in vaccine design away from live, attenuated, agents due to safety concerns, either
due to "vaccine breaks", incomplete attenuation, reversion, or amplification in
immunosuppressed patients, has also seen an accompanying decrease in vaccine
duration and efficacy. The use of killed agents, cloned recombinant proteins or
peptides requires large dosages and the presence of adjuvants. However, the long
term effects of such adjuvants have not been explored, and they have recently
been implicated as causative agents in vaccine induced sarcomas of cats (Hendrick
et. al., J. Am. Vet. Med. Assoc. 205 (1994), 1425-1429). Additionally, the
extracellular location of the injected antigen raises questions about the way in which
those antigens are presented to the immune system, and their appropriateness to
generate protection against naturally occurring infections.

Current immunization practices for canine parvovirus are marginal (Schultz, R.D.
(1994), The Challenge of Controlling a Newly Recognized Disease: Canine
Parvovirus Vaccines, IBC International Symposium, October 27-28, 108). Vaccines
consisting of either attenuated or killed organisms, must be given repeatedly to
create immunity, and immunization in the face of circulating maternal antibody titers
does not usually occur. This problem is compounded by the ability of maternal

antibody to inactivate vaccine while having been reduced to levels that are not protective leading to a "window of vulnerability" (Pollock and Carmichael, J. Am. Vet. Med. Assoc. 130 (1982), 37-42). The cloning of canine parvovirus has led to the development of two distinct vaccine strategies. The first was the introduction of the entire VP2 sequence into a baculovirus expression system. The protein product was harvested, and used to successfully immunize dogs (Turiso et. al., J. of Virology 66 (5) (1992), 2748-2753). The second strategy involves the subcloning or synthesis of peptide epitopes which are used to immunize dogs. These peptides have utilized the amino terminus sequence of VP2 (Casal et. al., 1995, loc. cit.) which resulted in successful immunization of dogs. The vaccines developed using these strategies have also been tested with mink enteritis virus, another closely related host range variant of parvovirus. Administration of either recombinant protein or peptide results in protective immunity to this commercially relevant disease of mink (Langeveld et. al., Vaccine 13(11) (1995), 1033-1037).

Modified live virus vaccines for feline panleukopenia are effective in protecting adult cats, but may produce birth defects in kitten embryos *in utero*, consequently they are not recommended for vaccinating intact female cats which could be pregnant. Because of the serious limitations of modified live virus vaccines, investigators began to explore the possibility of transfecting cells *in vivo* with genes expressing antigens from infectious organisms (reviewed in Donnelly et. al., J. Imm. Meth. 176 (1994), 145-152; Fynan et. al., Int. J. Immunopharmac. 17 (2) (1995), 79-83; Whalen et. al. (1995), DNA Mediated Immunization to the Hepatitis B Surface Antigen; Activation and Entrainment of the Immune Response in DNA Vaccines, New York: New York Academy of Sciences). Such a mechanism of immunization would imitate the pathway of viral gene expression without the attendant risk posed by attenuated organisms, while bypassing the need for typical adjuvants. The serendipitous discovery that intramuscular injection of "naked" plasmid DNA carrying a mammalian promoter would cause the DNA to be taken up by muscle cells and expressed (Wolff et. al., Science 247 (1990) 1465-1468), has led to a dramatic expansion of the new field of nucleic acid vaccination. Subsequent to the

original study, conditions affecting intramuscular injection of plasmid DNA have been further defined and broadened (Wolff et. al., Biotechniques 11 (1991), 474-485). The efficiency of transfer is relatively low, ranging from 1-5%, however that efficiency can be increased up to 40 fold by inducing muscle degeneration prior to the injection of plasmid DNA (Vitadello et. al., Hum. Gene. Ther. 5 (1994), 11-18; Danko and Wolff, Vaccine 12 (16) (1994), 1499-1502); Davis et. al., Hum. Gene. Ther. 4 (1993), 733-740). Two of the most commonly used myonecrotic agents are the local anesthetic bupivicaine, and cardiotoxin (Danko and Wolff, 1994, loc. cit.; Davis et. al., 1993, loc. cit.). A number of other techniques have been employed to transfer genes to muscle including retroviral vectors, adenoviral vectors, and liposomes. However, direct injection of naked DNA appears to be the most efficient of these delivery mechanisms at transferring and expressing foreign DNA (Davis et. al., 1993, loc. cit.).

Several routes of administration have been explored in addition to intramuscular injection. Common to all of these routes is the lack of a need for any agent or vector to facilitate the entry of the nucleic acid into target cells. Intravenous, intraperitoneal, intradermal, intranasal and subcutaneous injection of DNA plasmids have all resulted in immunization against influenza virus hemagglutinin (HA) in chickens (reviewed in Pardoll and Beckerkleg, Immunity 3 (1995), 165-169). Interestingly, these studies indicated that mucosal (intranasal) immunization with DNA did not produce the expected IgA response, but rather an IgG response, as seen with intramuscular injections. Additionally, intradermal immunization by bombardment with DNA coated gold microparticles was shown to be as efficacious as other methods of gene delivery at 100 to 1000 fold lower DNA concentrations than the other methods. Similar experiments have demonstrated that the intradermal, and intravenous routes result in immunization in mice and rabbits (Raz et. al., Proc. Natl. Acad. Sci. USA 91 (1994), 9519-9523). Emphasizing the simplicity of the approach, Raz and colleagues have indicated that there is the potential of bypassing the expensive and potentially cumbersome equipment required for tissue bombardment and intradermal immunization by coating a plastic

5

tuberculin PPD tine with DNA and scarifying the skin to achieve similar result to biolistic bombardment. DNA immunization has been tried with varying success in several mammalian species including cattle, swine, and non-human primates, predominantly by the intramuscular route.

The nature of the immune response to antigens present after intramuscular injection of DNA expression constructs has been reported to involve both the humoral and cellular arms of the immune system. When reporter DNA constructs are injected, they appear to be contained within mature myofibers. This is supported by the evidence that muscle regeneration appears to increase the efficiency of plasmid expression. Antigen presentation might occur by MHC class I presentation by the muscle cell, antigen uptake from myocytes by bone marrow derived antigen presenting cells (APCs) or by direct DNA transfection of APCs that are migrating through muscle (reviewed in Pardoll and Beckerleg, 1995, loc. cit.; Whalen et. al., 1995, loc. cit.). Simple antigen presentation by myocytes would seem unlike to be completely responsible for this phenomenon for two reasons. First is the growing body of evidence that presentation of antigen in the absence of co-stimulatory signals such as B7 and B7-2 results in tolerization to antigens (Chen and Nabavi, Immunity 1 (1994), 147-154). Secondly, muscle has been reported to have extremely low levels of expression of MHC class I (Karpati et. al., Ann. Neurol. 23 (1988), 64-72). Therefore, it seems likely that antigen presentation is performed by professional APCs that have acquired antigen either by scavenging it or through direct acquisition and expression of the transferred gene (Pardoll and Beckerleg, 1995, loc. cit.; Whalen et. al., 1995, loc. cit.). An experiment performed with biolistic particle bombardment in the mouse would seem to favor the latter approach. Mice were bombarded in the pinna and the ear surgically removed five minutes after bombardment. These mice retained the ability to form an immune response, and that response did not differ qualitatively from that induced by bombardment alone. Clearly, a highly mobile cell population must be responsible for initiating and maintaining the immune response in these mice. The dendritic cell has been proposed as this cell (Fynan et. al., 1995, loc. cit.). Significantly, the

6

character of the immune response induced by particle bombardment differs slightly from the response resulting from intramuscular injection, with IgG1 predominating in intramuscular injections while IgG2 predominates in intradermal injections. The primary reason for this difference may rest in the myocytes, which in intramuscular immunization, continue to express antigen for periods exceeding one month (Pardoll and Beckerleg, 1995, loc. cit.). This continuous expression results in what has been called immune entrainment (Whalen et. al., 1995, loc. cit.). The practical result of this phenomenon is that muscle expression acts as a continuous boost. Frequently, titers from a single injection may rise over a period of 4 to 8, or even more weeks. Classic booster responses have also been observed with injections of DNA given over 6 months after the primary injection. Curiously, DNA booster injections, given during the period of rising antibody titers, may have no effect, or may even reduce the response to antigen (Davis et al., "Introduction of systemic and mucosal immunity to HBV with plasmid DNA", international Meeting on Nucleic Acid Vaccines, February 5-7, 1996, Bethesda, Maryland, USA).

The ability to generate a measurable immune response is not a priori sufficient for vaccination. Rather the immune response must contain the appropriate elements to protect the host from infection, invasion, and disease. Therefore, protection against infection in challenge studies remains the single most convincing demonstration of vaccine efficacy. DNA based vaccines have been able to protect chickens against lethal influenza virus challenges (Robinson et. al., Vaccine 9 (1993), 957-960) and mice against Mycoplasma pulmonis infection (Lai et. al., DNA and Cell Biology 14(7) (1995), 643-651). Additionally, mice could be protected against the establishment of persistent infections of lymphocytic choriomeningitis virus by DNA immunization (Martins et. al., J. of Virology 69(4) (1995), 2574-2582).

The prior art, with respect to nucleic acid vaccination as related to the present invention is summarized in the following articles: Donnely et al., 1994, loc. cit., Fynan et al., 1995, loc. cit., Whalen et al., 1995, loc. cit. as regards nucleic acid

vaccination, and Turiso et al., 1992, loc. cit. and Casal et al., 1995, loc. cit., as regards parvoviral vaccination.

The above recited prior art only tested the immune response of mice to vaccination with nucleic acids. As is well known in the art, experimental results obtained with mice cannot be transferred to animals like cats, dogs and mink due to the taxonomic difference between these animals. Since, on the other hand, no suitable vaccines against parvoviral infections of canids, felids and mustelids could be provided by the prior art, the technical problem underlying the present invention was to provide such a vaccine. Said vaccine should effectively protect in particular dogs, cats and mink from the potentially life threatening effect of parvovirus infections. The solution to the above technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to an anti-parvovirus vaccine comprising nucleic acid molecules encoding at least one parvovirus-specific epitope and a pharmaceutically acceptable carrier.

Surprisingly, in accordance with the invention it has now been found that immunization with "naked" DNA that encodes at least one parvovirus-specific epitope yields an immune response that protects the vaccinated animal from parvovirus-induced disease upon subsequent parvoviral infection.

In a preferred embodiment of the anti-parvovirus vaccine of the invention, at least one of said parvovirus-specific epitopes is a T-cell epitope.

The person skilled in the art, when designing a protective vaccine in the light of the teachings of the present invention, is able to devise a vaccine comprising a nucleic acid sequence encoding only a T-cell epitope to be used in the vaccine of the invention.

Similarly, the person skilled in the art is able to devise a vaccine comprising only a B-cell epitope encoding DNA for use in a nucleic acid vaccine according to the

present invention. Therefore, in a further preferred embodiment, the anti-parvovirus vaccine comprises at least one B-cell epitope. Alternatively, nucleic acids encoding said at least one B-cell epitope can be comprised in said vaccine together with nucleic acids encoding at least one T-cell epitope. T- and B- cell epitopes may be encoded by the same or different DNA molecules.

In a further preferred embodiment of the anti-parvovirus vaccine according to the invention, said nucleic acid molecules are DNA molecules or RNA molecules. The term "DNA molecules" is used here in its broadest sense and includes e.g. cDNA molecules, genomic DNA, synthetic and semisynthetic DNA molecules. Equally, the term "RNA molecules" is used in its broadest possible sense herein.

In a further preferred embodiment of the anti-parvovirus vaccine according to the invention, said nucleic acid molecules encode the parvoviral VP1 and/or VP2 nucleocapsid proteins.

The parvoviral genome is approximate 5000 base pairs long, and contains approximately four open reading frames. Two of these open reading frames encode, VP1 and VP2, the major capsid proteins of parvovirus. VP2 is contained within VP1 and consist of all but a small portion of the 5' end of the VP1 gene or the amino terminus of the VP1 protein. The sequence exclusive to VP1 is known to encode an epitope which is responsible for stimulating a T-cell response. The sequence common to VP1 and VP2 encodes at least one epitope which stimulates an antibody response. Natural infections with parvoviruses generate significant protective responses within one week of infection. Expression of the entire VP1 gene or the epitope within VP1 (and VP2) also results in protective immunity through both the cellular and humoral arms or the immune system.

Accordingly, the person skilled in the art may use nucleic acid sequences comprising either the sequence encoding the VP1 epitope as a representative of a parvoviral T-cell epitope or a nucleic acid sequence encoding the VP2 epitope as a representative of a parvoviral B-cell epitope or a combination of both for the preparation of a vaccine for immunizing canids, felids or mustelids.

In a particularly preferred embodiment of the vaccine of the invention, said nucleic acid molecules comprise the complete VP1 open reading frame, i.e. the reading frame encoding the VP1 nucleocapsid protein. In the case of the canine parvovirus, the complete length of the open reading frame is 2169 base pairs. Said orf is preferably expressed under the control of a suitable foreign promoter.

A further preferred embodiment of the vaccine of the invention, said epitope is derived from a parvovirus-genome, said parvovirus being capable of infecting canids, felids or mustelids and preferably dogs, cats or mink. Due to the strong overall homology of the genome of parvoviruses infecting either canids, felids or mustelids, a vaccine derived from a nucleic acid derived from either canids, felids or mustelids can be used to successfully immunize any of the other groups of animals mentioned.

In an additional preferred embodiment of the vaccine of the present invention, said nucleic acid molecules encoding at least one parvovirus-specific epitope are comprised in an expression vector, said expression vector being functional in mammalian cells.

To the person skilled in the art, numerous suitable expression vectors for carrying out the present invention are available. Accordingly, the vectors recited in the attached examples as a starting basis for developing the vaccine of the present invention are not in any way intended to limit the scope of the present invention. Further suitable vectors are available from commercial companies including Invitrogen, Vical, and Agracetus and can be readily produced by the person skilled in the art.

An additional preferred embodiment of the invention relates to an anti-parvovirus vaccine, wherein said vaccine comprises or said expression vector encodes at least one additional antigen.

Said additional antigen may serve the function to enhance the immune response to the parvovirus-specific epitope. In this regard, said additional antigen has the function of a carrier protein. Alternatively, the different antigen may induce an immune response to a different pathogen and thus serve the function of creating a multivalent vaccine. It is also possible that the additional antigen serves both functions.

There are multiple ways to devise a vector that encodes at least one parvovirus-specific epitope as well as at least one additional antigen. Thus, for example, the nucleic acid encoding the additional antigen may be cloned into the nucleic acid sequence encoding the at least one parvovirus-specific epitope. If no suitable restriction site is located within said nucleic acid sequence encoding said parvovirus-specific epitope, such a restriction site can be generated using conventional methods. Conversely, the nucleic acid sequence encoding said at least one parvovirus-specific epitope may be cloned into a DNA sequence encoding said at least one additional antigen. The resulting polypeptide may be a fusion protein. Alternatively, the at least one parvovirus-specific epitope and the at least one additional antigen may be expressed as separate proteinaceous entities.

It is clear from the statements made in the previous paragraph that a particular preferred embodiment of the invention relates to a vaccine, wherein said additional antigen is an immunogen.

An example of such an immunogen which has turned out to be effective as a carrier molecule, is the hepatitis B-surface antigen, and preferably the human hepatitis B-surface antigen pre-S2 protein.

An additional particularly preferred embodiment of the invention relates to a vaccine comprising the recombinant vector pGT36VP1. Said vector comprises a nucleic acid molecule that represents the complete VP1 open reading frame. The detailed construction of said vector is described in Example 2.

In a further particularly preferred embodiment of the vaccine of the present invention, said nucleic acid molecule encoding at least one T-cell epitope is selected from the group of epitopes encoded by the degenerate nucleic acid sequence

CCNAARATHTTYATHAAYYTNGCNAARAARAARAARGCNGGC;

wherein N denotes any base, R denotes purine, Y denotes pyrimidine and H denotes A, C or T;

and is preferably the epitope encoded by the nucleic acid sequence

CCGAAAATATTCATCAACCTGGCTAAGAAGAAGAAAGCTGGC.

In a further particularly preferred embodiment of the vaccine of the present invention, said nucleic acid molecules encoding at least one B-cell epitope is selected from the group of epitopes encoded by the degenerate nucleic acid sequence

TSNGAYGGNGCNGTNCARCCNGAYGGNGGNCARCCNGCNGTNMGN;

wherein N denotes any base, R denotes purine, Y denotes pyrimidine, S denotes G or C and M denotes C or A;

and is preferably the epitope encoded by the nucleic acid sequence

TCAGACGGTGCTGTACAGCCAGATGGAGGACAACCCGCGGTTCGC.

In an additional preferred embodiment, the vaccine of the present invention comprises a mixture of nucleic acids encoding both T-cell and B-cell epitopes.

12

An example of this embodiment of the invention is the case that any of the previously mentioned nucleic acid molecules encoding said at least one B- and T-cell epitopes are comprised in different expression vectors.

In a further preferred embodiment of the anti-parvovirus vaccine of the invention, said vaccine additionally comprises an adjuvant.
Adjuvants for immunization are well known in the art and suitable adjuvants can be combined with the nucleic acid sequences described in the previously mentioned embodiments for the formulation of a vaccine by the person skilled in the art.

A particularly preferred embodiment of the present invention relates to a vaccine, wherein said adjuvant is a DNA molecule comprising unmethylated CpG motifs.
DNA has long been known to contain the genetic information that makes each individual unique. In addition to this role as blueprint of the cell, Dr. Arthur Krieg has recently discovered a short stretch of DNA, called a CpG motif, that causes potent immune activation (Nature (1995), Vol 374, 546). DNA with CpG motifs ("CpG DNA") can be synthesized easily and inexpensively, and used to selectively activate the immune system. Therapeutic applications for CpG DNA-mediated immune activation include increasing the effectiveness of vaccines, helping the immune system to destroy cancer, and preventing or treating infection. DNA is a natural part of the body, and may be much safer than drugs now used to enhance the immune system; see patent application U.S. Serial No. 08/386,063; 08/461,036; 08/462,799.
This invention combines for the first time the use of plasmid DNA as a vaccine together with CpG containing oligonucleot s functioning as adjuvant for the DNA based vaccine.
The superior immunological properties of this vaccine is described in the attached examples.

Particularly preferred is an anti-parvovirus vaccine, wherein said DNA molecule comprising unmethylated CpG motifs is


TCCATGACGTTCCTGATGCT.


In a further particularly preferred embodiment of the anti-parvovirus vaccine of the invention, said DNA molecule comprising unmethylated CpG motifs comprised a phosphorothioate-modified backbone.

The phosphorothioate modification is particularly advantageous since it appears to further enhance the adjuvant capacities of the unmethylated CpG motifs; see, e.g. Krieg et al., loc. cit.


With the vaccine according to the present invention it is possible to obtain an effective immunization even if the vaccine is only applied once.


Additionally, the present invention relates to the use of an anti-parvovirus vaccine of the invention for the vaccination of canids, felids or mustelids, a method for vaccinating canids, felids or mustelids by administering a suitable dose of the vaccine of the invention to an animal in need thereof, as well as to the use of the ingredients of the vaccine of the invention mentioned hereinabove, such as nucleic acids specified in the various embodiments or adjuvants, such as the unmethylated CpG motifs, or any combination thereof for the preparation of a vaccine for the immunization of canids, felids or mustelids against parvoviral disease.


Preferably, said canids, felids or mustelids are dogs, cats or mink. The person skilled in the art, here, a veterinarian, is perfectly aware of which dose and application route should be applied. Additionally he is familiar with the number of vaccine applications as well as the optimal time span between primary and booster vaccinations.

In a particularly preferred embodiment the vaccine according to the invention is used for a vaccination protocol in which the vaccine is only applied once and leads to a sufficient immunization ("single dose immunization").

The vaccine of the invention is preferably administered by one of the following routes: intramuscular by needle and syringe, intramuscular by Biojector 2000®, intradermal by needle and syringe, intradermal by particle bombardment, intradermal by scarification, intravenous or intraperitoneal.
The examples illustrate the invention.

15

## EXAMPLE 1: SYNTHESIS OF ANTIGENIC EPITOPES OF THE FELINE PARVOVIRUS SUBGROUP

Synthetic deoxyribonucleic oligonucleotides were produced with the sequence

AATTCCCCGAAAATATTCATCAACCTGGCTAAGAAGAAGAAAGCTGGC

and the complementary strand

TCGAGCCAGCTTTCTTCTTCTTAGCCAGGTTGATGAATATTTTCGGGG

which when annealed to each other contain overhangs which will bind to an Eco RI and Xho I restriction site, and in the proper reading frame, encode the T-cell epitope of VP1, PKIFINLAKKKKAG. These oligonucleotides were ligated into the Eco RI and Xho I sites in the pre S2 region of Human Hepatitis B Surface antigen in the plasmid pCMV-S2S (Michel et al., Proc. Natl. Acad. Sci. USA 92 (1995), 5307-5311) in the process deleting the majority of the pre S2 region. The resulting construct was termed pCMVS-VP1e. The RNA transcript produced from the CMV promoter in this construct contains an open reading frame with an internal start codon (AUG). When the first ATG is used, the VP1 epitope, part of the pre S2 region and all of the S region are produced as a single protein. The internal AUG produces only the S protein. Similarly, deoxyribonucleic oligonucleotides with the sequence

AATTCAGACGGTGCTGTACAGCCAGATGGAGGACAACCCGCGGTTCGC

and the complementary strand

TCGAGCGAACCGCGGGTTGTCCTCCATCTGGCTGTACAGCACCGTCTG

which encode the peptide epitope SDGAVQPDGGQPAVR, from VP2, were
cloned into pCMV-S2S in the same manner as the VP1 epitope. The resulting
plasmid was named pCMVS-VP2e. Both of the DNA sequences encoding these
epitopes are artificial sequences, and do not represent the DNA sequence from
naturally occurring parvoviruses.

## EXAMPLE 2: CLONING THE VP1-VP2 GENE OF CANINE PARVOVIRUS AND CONSTRUCTION OF PLASMIDS EXPRESSING PARVOVIRUS GENES

The entire coding region of canine parvoviral VP1 (VP1 and VP2) was cloned by
polymerase chain reaction amplification from tissue culture supernatants
containing CPV type 2a. The primers were located just outside the open reading
frame for VP1, included Not I (downstream) and BamH I (upstream) restriction
sites and had the following sequences:

cgg gat ccG AGA CGA CTT GGA TTA AGG TA for the 5' or upstream primer, and

gtg cgg ccg CTA GTT GAT ATG TAA TAA AC for the 3' or downstream primer.

The PCR product was sequentially restricted with Not I and BamH I and ligated
into similarly digested bacterial expression plasmid pGT36 (Conry et al, Cancer
Gene Therapy 2(1) (1995), 33-38 in the sense orientation. Orientation and
sequence of VP1 (and VP2) were confirmed by DNA sequencing of the entire
VP1 insert. This DNA cloned will be referred to as pGT36VP1.

## EXAMPLE 3: EXPRESSION OF THE CLONED PARVOVIRUS VP1-VP2 GENE IN CULTURED CELLS

A murine fibroblast (NIH 3T3) was transfected with the pGT36VP1 clone. Cells
were then examined for the presence of immunoreactive canine parvoviral

proteins by immunofluorescent assay with a canine antiserum to canine parvovirus. The pattern of staining was indistinguishable to cells infected with canine parvovirus, while negative controls (pGT36, no DNA) showed no staining, indicating that the pGT36VP1 clone produced a protein product recognized by anti-parvoviral antibodies.

## EXAMPLE 4: IMMUNIZATION OF DOGS WITH PARVOVIRUS (EPITOPE) NUCLEIC ACID VACCINES

Three 11 week old puppies were immunized with 150 µg pCMVS-VP1e and 100 µg of the synthetic phosphorothioate oligonucleotide TCCATGACGTTCCTGATGCT (ISO) in the right quadricepts muscle, three 11 week old puppies were immunized with 150 µg pCMVS-VP2e and 100 µg of the ISO, and three 11 week old puppies were immunized with 150 µg pCMVS-VP1e, 150 µg pCMVS-VP2e, and 100 µg of the ISO. The puppies were boosted 3 weeks later with identical doses, and monitored for the development of antibody titers to the carrier protein, Hepatitis B surface antigen, and the canine parvoviral epitopes. A significant antibody response was seen to both hepatitis and canine parvovirus in the animals in the pCMVS-VP1e plus pCMVS-VP2e, and the pCMVS-VP2e groups. No antibody response was expected to canine parvovirus in the pCMVS-VP1e as this represents a T-cell epitope. This expectation was confirmed experimentally. The response was greatest in the pCMVS-VP1e plus pCMVS-VP2e indicating that the multivalent vaccine was more effective.

18

# EXAMPLE 5: IMMUNIZATION OF ANIMALS WITH A NUCLEIC ACID EXPRESSING PARVOVIRUS VP1 AND VP2 GENES

(a)    One of three Balb-c mice immunized with pGT36VP1 demonstrated an immune, in particular an antibody response to canine parvovirus one week after particle bombardment with 1 µg of the plasmid.

(b)    Three juvenile (approximate age 6 months) beagle dogs with no anti-canine parvovirus antibody titers were immunized with 150 µg of pGT36VP1 and 100 µg of the ISO in the right quadriceps muscle. Significant anti-canine parvovirus antibody responses developed beginning within 2 weeks of immunization in one animal, and continuing to increase for at least 3 weeks in all three animals. A control beagle, injected with the vector DNA and ISO showed no increase in antibody titer.

(c)    Six juvenile dogs with no anti-canine parvovirus antibody titers were immunized with 400µg of pGT36VP1 in the right quadriceps muscle. Three of these dogs received an additional 400µg of DNA from *E. coli* as an immunostimulatory adjuvant, which was coadministered with pGT36VP1. All six dogs showed anti-parvovirus antibody response within two weeks of immunization, which reached titers significantly above that known to be protective in 3 to 5 weeks. The three dogs receiving immunostimulatory DNA reached higher antibody titers, more rapidly than the other dogs, indicating a positive CpG immune stimulatory effect.

# EXAMPLE 6: PROTECTION OF DOGS, IMMUNIZED WITH A NUCLEIC ACID VACCINE EXPRESSING PARVOVIRUS VP1 AND VP2 GENES, FROM CHALLENGE INFECTION OF CANINE PARVOVIRUS

(a)    The three beagle dogs immunized in example 5 were challenged with a
       standard tri-valent dose of canine parvovirus, used in parvoviral vaccine
       trials, via the intranasal route.   None of the dogs showed any signs of
       parvoviral related illness indicating protection from disease.   Two dogs
       shows slight increase in anti-canine parvovirus antibody responses,
       consistent with the pre-challenge trend, with no fecal shedding of
       parvovirus, indicating complete protection from infection. The control
       beagle showed signs of parvoviral disease following challenge,
       accompanied by a dramatic increase of anti-canine parvovirus antibody
       response.

(b)    Four juvenile dogs with no anti-canine parvovirus antibody titers were
       immunized intramuscularly with doses of pGT36VP1 including 200, 400,
       600, and 800μg of plasmid. All dogs developed anti-canine parvovirus
       antibodies within one week, which peaked to protective titers within 2
       weeks. A negative control dog injected with saline showed no anti-canine
       parvovirus antibody. The dog receiving 600μg of plasmid DNA sustained
       an antibody titer longer than the other dogs. All dogs were reinjected 16
       weeks after the initial injection with the same preparation and dose. All
       dogs except the negative control and 200μg dose showed an anamnestic
       antibody response. All dogs except the negative control, but including the
       200μg dose showed serum neutralizing antibody titers after reinjection
       known to be protective. All dogs were challenged at 24 weeks after initial
       injection with virulent street virus containing three virus isolates known to
       cause disease in unimmunized dogs. All dogs immunized with pGT36VP1
       nucleic acid vaccine were protected from infection and disease. The
       negative control dog had clinical disease typical of canine parvovirus and
       shedded virus in the stool. This experiment demonstrates that doses of
       pGT36VP1 ranging from 200 to 800μg of plasmid, given as a primary and

booster injection stimulated production of antibody titers and protected against virulent challenge.

(c)    Three juvenile dogs with no anti-canine parvovirus antibody titers were injected with 150µg of pGT36VP1 plus 150µg of oligonucleotide DNA synthesized to contain optimal CpGs. All dogs developed anti-canine parvovirus antibody and were protected from challenge with virulent virus. This experiment demonstrates that immunization with a lower dose of pGT36VP1 will confer protective immunity when immunization is amplified with immunostimulatory oligonucleotides.

(d)    Six juvenile dogs with no anti-canine parvovirus antibody titers were injected with a single dose of 200 or 800µg of pGT36VP1. All dogs developed anti-canine parvovirus antibody titers initially, which decayed to undetectable levels by 12 weeks post immunization. Without giving a booster immunization, all dogs were challenged with virulent virus and none became infected or ill. This experiment demonstrates that protective immunity is achieved after a single dose of pGT36VP1 and this protection is independent of the presence of measurable anti-canine parvovirus antibody. This result can be interpreted as resulting from cellular immunity conferred by long lived immunological memory T-cells.

These studies demonstrate that selected DNA sequences from the feline parvovirus subgroup when inserted into a mammalian expression plasmid can transfect cells in culture and in animals. Furthermore, it was demonstrated that our DNA expression plasmids can invoke an antibody response in animals which may lead to protection against virulent viral challenge.

# CLAIMS

1.    An anti-parvovirus vaccine comprising nucleic acid molecules encoding at least one parvovirus-specific epitope and a pharmaceutically acceptable carrier.

2.    The anti-parvovirus vaccine according to claim 1, wherein at least one of said parvovirus-specific epitopes is a T-cell epitope.

3.    The anti-parvovirus vaccine according to claim 1 or 2, wherein at least one of said parvovirus-specific epitopes is a B-cell epitope.

4.    The anti-parvovirus vaccine according to any one of claims 1 to 3, wherein said nucleic acid molecules are DNA molecules or RNA molecules.

5.    The anti-parvovirus vaccine according to any one of claims 1 to 4, wherein said nucleic acid molecules encode the parvoviral VP1 and/or VP2 nucleocapsid proteins.

6.    The anti-parvovirus vaccine according to claim 5 wherein said nucleic acid molecules comprise the complete VP1 open reading frame.

7.    The anti-parvovirus vaccine according to any one of claims 1 to 6 wherein said epitope is derived from a parvovirus-genome, said parvovirus being capable of infecting canids, felids, or mustelids and preferably dogs, cats or mink.

8.    The anti-parvovirus vaccine according to any one of claims 1 to 7, wherein said nucleic acid molecules encoding at least one parvovirus-specific

22

epitope are comprised in an expression vector, said expression vector being functional in mammalian cells.

9.     The anti-parvovirus vaccine according to any one of claims 1 to 8, wherein said vaccine comprises or said expression vector encodes at least one additional antigen.

10.    The anti-parvovirus vaccine according to claim 9, wherein said additional antigen is an immunogen.

11.    The anti-parvovirus vaccine according to any one of claims 8 to 10, wherein said expression vector comprising said nucleic acid molecules encoding at least one parvovirus-specific epitope is pGT36VP1, the construction of which is described in Example 2.

12.    The anti-parvovirus vaccine according to any one of claims 2 to 11, wherein said nucleic acid molecule encoding at least one T-cell epitope is selected from the group of epitopes encoded by the degenerate nucleic acid sequence

CCNAARATHTTYATHAAYYTNGCNAARAARAARAARGCNGGC;

wherein N denotes any base, R denotes purine, Y denotes pyrimidine and H denotes A, C or T;

and is preferably the epitope encoded by the nucleic acid sequence

CCGAAAATATTCATCAACCTGGCTAAGAAGAAGAAAGCTGGC.

13.    The anti-parvovirus vaccine according to any one of claims 3 to 12, wherein said nucleic acid molecules encoding at least one B-cell epitope is

selected from the group of epitopes encoded by the degenerate nucleic acid sequence

TSNGAYGGNGCNGTNCARCCNGAYGGNGGNCARCCNGCNGTNMGN;

wherein N denotes any base, R denotes purine, Y denotes pyrimidine, S denotes G or C and M denotes C or A;

and is preferably the epitope encoded by the nucleic acid sequence

TCAGACGGTGCTGTACAGCCAGATGGAGGACAACCCGCGGTTCGC.

14. The anti-parvovirus vaccine according to any one of claims 1 to 13 comprising a mixture of nucleic acids encoding both T-cell and B-cell epitopes.

15. The anti-parvovirus vaccine according to any one of claims 1 to 14 additionally comprising an adjuvant.

16. The anti-parvovirus vaccine according to claim 15, wherein said adjuvant is a DNA molecule comprising unmethylated CpG motifs.

17. The anti-parvovirus vaccine according to claim 16, wherein said DNA molecule comprising unmethylated CpG motifs is

TCCATGACGTTCCTGATGCT.

18. The anti-parvovirus vaccine according to claim 16 or 17, wherein said DNA molecule comprises a phosphorothioate-modified backbone.

24

19.  Use of the anti-parvovirus vaccine according to any one of claims 1 to 18 for the vaccination of canids, felids or mustelids.

20.  The use according to claim 19, wherein said canids, felids or mustelids are dogs, cats or mink.

21.  The use according to claim 19 or 20, wherein said vaccine is administered by one of the following routes: intramuscular by needle and syringe, intramuscular by Biojector 2000®, intradermal by needle and syringe, intradermal by particle bombardment, intradermal by scarification, intravenous or intraperitoneal.

22.  The use according to claim 19 or 20, wherein immunization is achieved by a single application of the vaccine.

# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/35    A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N  A61K  C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | H.C.J. ERTL ET AL: "Genetic Immunization" VIRAL IMMUNOLOGY, vol. 9, no. 1, January 1996, pages 1-9, XP000670099 --- | 1-22 |
| A | EP 0 647 655 A (INMUNOLOGIA & GENETICA APLICADA S.A.) 12 April 1995 see the whole document --- | 1-22 |
| A | J.P.M. LANGEVELD ET AL.: "Effective induction of neutralizing antibodies with the amino terminus of VP2 of canine parvovirus as a synthetic peptide" VACCINE, vol. 12, no. 15, 1994, GUILDFORD GB, pages 1473-1480, XP002040911 see the whole document ----- | 1-22 |

☐ Further documents are listed in the continuation of box C.      ☒ Patent family members are listed in annex.

* Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

'&' document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 25 September 1997 | 3 0. 09. 97 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. ( + 31-70) 340-2040, Tx. 31 651 epo nl, Fax ( + 31-70) 340-3016 | Cupido, M |

# INTERNATIONAL SEARCH REPORT

**Box I    Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

   **see FURTHER INFORMATION sheet PCT/ISA/210**

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II    Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**        [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

FURTHER INFORMATION CONTINUED FROM     PCT/ISA/   210

Remark : Although claims 19-22 are directed to a method of treatment of
the animal body , the search has been carried out and based on the
alleged effects of the composition.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| EP 0647655   A | 12-04-95 | ES   2065254 A | 01-02-95 |
| | | ES   2089966 A | 01-10-96 |
| | | AU    680264 B | 24-07-97 |
| | | AU   7004894 A | 15-08-94 |
| | | CA   2132742 A | 24-07-94 |
| | | WO   9417098 A | 04-08-94 |
| | | NZ    259882 A | 24-03-97 |

THIS PAGE BLANK (USPTO)